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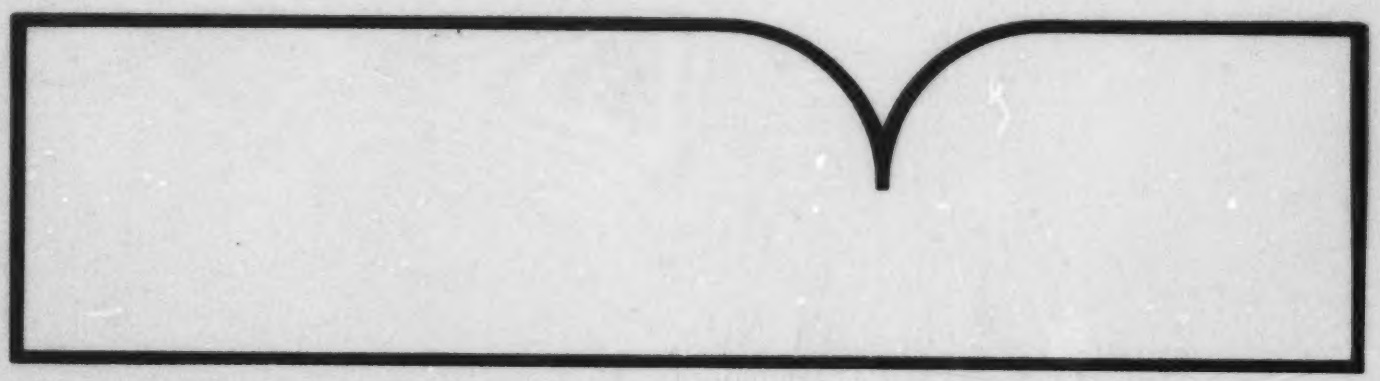

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JOURNAL ARTICLE

Investigation of HCB as a Metabolite from Female Rats Treated Daily for Six Days with Lindane

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Abstract

The biotransformation of lindane to hexachlorobenzene (HCB) by male rats was recently reported. Since HCB has been widely detected in human milk samples, and since the transplacental transfer of HCB to the fetus has been demonstrated in several species, the metabolism of lindane to HCB in female rats was investigated. Young adult female Fischer 344 rats were dosed p.o. with either 20 mg lindane/kg/day or an equivalent volume of the peanut oil vehicle. Feces samples were collected daily for two consecutive 4-hr intervals and a 16-hr interval. Twenty-four hours after the final treatment, all rats were sacrificed and adipose tissue samples were excised at necropsy. Extracts of fat and feces samples were analyzed by gas-liquid chromatography (GLC) on column packings of different polarity. Results of this study indicated that no significant biotransformation of lindane to HCB occurred in the female Fischer 344 rat.

Introduction

Recently it was reported that the organochlorine insecticide lindane, γ-hexachlorocyclohexane (γ-HCH) was metabolized by rats to hexachlorobenzene (HCB) (1). Considering the widespread presence of HCB in human milk samples (2-7) and the reported transplacental transfer of HCB from pregnant mice (8), rats (8,9), and rabbits (10), metabolism of lindane to the less biodegradable HCB could represent a potential hazard to both mothers and their developing offspring.

The investigation by Gopalaswamy and Aiyar, which reported the biotransformation of γ-HCH to HCB, employed male rats and Aroclor 1254 to induce microsomal enzyme activity (1). In the work reported here, young female rats were treated daily with 20 mg lindane/kg for six days, both to induce microsomal enzyme activity (11-15) and to determine whether significant biotransformation of lindane to HCB occurred.

Experimental

Apparatus

A model MT-220 gas chromatograph (Tracor) with a ^{63}Ni electron capture detector (ECD) and linearizer was employed for the analysis of tissue extracts. The output from the gas chromatograph was processed by a model 812 Analytical Processor (Tracor Instruments). The columns used consisted of 183-×0.63-cm o.d. glass U-tubes, packed with either 1.5% OV-17 + 1.95% QF-1 on 80/100 mesh Gas Chrom Q (Applied Science Laboratories, Inc.) or 5% EGS + 1% H_3PO_4 on 80/100 mesh Anakrom A (Analabs). Both columns were maintained isothermally at 160°C. Inlet and detector temperatures were 250° and 350°C, respectively. The 5% methane/argon carrier gas flow rate was regulated at 55 cm³/min.

A Meyer model 112 N-Evap anaytical evaporator (Organomation) was employed to concentrate tissue extracts under N_2 prior to cleanup.

A Stir-R model K43 stirring device (TRI-R Instrument) was used as an aid in tissue grinding operations.

A Vortex test tube mixer (Scientific Industries Inc.) was used in the extraction of tissue samples.

After extraction of tissue samples, the aqueous and organic layers were separated by centrifugation at 3000 rpm on a model RC2-B refrigerated centrifuge (Ivan Sorvall, Inc.).

Glassware included 300-mm × 25-mm o.d. K-420530 Chromaflex columns and K-885450 Dual Tissue Grinders (Kontes) Kimble 45066 round bottom, screw-capped tubes (Fisher Scientific) and 25-mL graduated K-570050 concentrator tubes (Kontes).

MicroFlorisil columns were packed with 16 g of 100 mesh Florisil, topped with 1.6 g anhydrous Na_2SO_4. The packed columns were washed with 50 mL of benzene followed by 50 mL of methanol and were stored in a 130°C oven for at least 24 hr before use.

Reagents and Materials

Hexachlorobenzene, +99% and lindane, +99%, were obtained from the Environmental Protection Agency, Environmental Monitoring Systems Laboratory, Pesticides and Industrial Chemicals. Certified A.C.S. anhydrous, granular Na_2SO_4 (Fisher Scientific Co.) was extracted with methylene chloride and oven-dried at 130°C. Water used for the 2% Na_2SO_4 solution was pre-extracted with benzene. Florisil (Floridin Co.), 100 mesh,

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activated at 1200°F was employed in the cleanup of the tissue extracts. All solvents were pesticide quality or better.

Methods

Nine young adult female Fischer 344 rats (105 to 120 g) were individually housed in stainless steel metabolism cages and randomly assigned to treatment or control groups. Six rats were dosed perorally with 20 mg lindane/kg/day for six days in a volume of peanut oil equivalent to 0.1% of the body weight of the animal. Control animals received the same volume of unadulterated vehicle for six days. Feces were collected each day after two consecutive 4-hr intervals and a 16-hr interval and were then stored at −65°C until analysis. Use of short collection intervals was intended to reduce potential microbial biotransformation of lindane. Twenty-four hours after the final treatment, all rats were sacrificed, and at necropsy, fat samples were excised, extracted with benzene, and the extracts were analyzed for pesticide content after column cleanup.

Since it has been reported that rats accumulated HCB in fat and excreted the chemical primarily in feces (16), both fat and feces from the final day of this study were extracted and analyzed to determine the extent of metabolism of lindane to HCB.

For each rat, approximately 500 mg fecal samples from the combined 4-hr collection intervals on the final day of treatment were extracted by the method described in the EPA's *Manual of Analytical Methods* (17). The samples were macerated in micro tissue grinders with 2.5 mL of acetonitrile, centrifuged at 300 rpm, and the supernatant transferred to 25-mL round bottom, screw-capped tubes. After two more extractions, 10 mL of aqueous 2% Na_2SO_4 was added to the combined acetonitrile fractions. This aqueous acetonitrile mixture was then extracted with 2 mL of benzene by vortexing the mixture for 2 min, centrifuging the mixture at 3000 rpm, and transferring the benzene to a 15-mL graduated test tube. This procedure was repeated twice and the combined benzene extracts were concentrated under N_2 to about 0.5 mL in the analytical evaporator.

The concentrated samples were quantitatively transferred to microFlorisil columns which had been prewet with 10 mL benzene. After transfer of the samples to the columns, 12 mL of benzene was added and the eluate was collected in 25-mL graduated concentrator tubes. This elution with benzene was followed by 12 mL of 1% methanol in benzene, and the combined eluant was diluted to 25 mL with benzene for analysis. It was determined that further elution with another 12 mL of 1% methanol in benzene did not improve recovery of either hexachlorobenzene or lindane.

To determine the recovery of HCB and γ-HCH by this procedure, an acetonitrile solution containing 23.72 ng HCB + 40.68 ng γ-HCH/mL was prepared. Control feces samples were macerated with 2.5 mL of this solution followed by two consecutive macertions with 2.5-mL portions of unadulterated acetonitrile. The combined acetonitrile extracts were diluted with aqueous 2% Na_2SO_4, extracted with benzene, and cleaned up as described above.

Approximately 500-mg samples of adipose tissue were macerated three times with 3-mL portions of benzene. After centrifugation at 3000 rpm and transfer of the benzene to glass-stoppered 15-mL graduated tubes, the combined benzene extracts were diluted with benzene to 10 mL. Lipid content was determined on 5 mL of the benzene extract by evaporating the solvent and weighing the residue. The remaining 5 mL of benzene was concentrated and eluted from a microFlorisil column as described above.

During analysis, the analytical processor detected a peak in the fecal extracts which it subsequently identified as HCB on the 1.5% OV-17 + 1.95% QF-1 column but not on the 5% EGS column. To further investigate this discrepancy, all samples were reanalyzed on both columns after addition of either standard HCB solution or an equal volume of solvent. To 1-mL aliquots of each sample, 0.5 mL of an isooctane solution containing 1.18 ng HCB/mL was added. To a second 1-mL aliquot, 0.5 mL of isooctane was added. These samples were then chromatographed back to back for comparison.

Student's t-test (18) was used as an aid in the interpretation of the data in this study. Comparisons were considered significantly different at $p < 0.05$.

Results and Discussion

Recovery from fecal samples ranged from 78.9 to 92.1% using the procedures described in the Experimental section (Table I).

Comparative retention times for HCB and lindane on the very polar EGS column and the slightly polar mixed phase column are presented in Table II. While the retention time of HCB was about 1 min shorter on the EGS column (3.14 vs. 4.39 min), the retention time of lindane on this column packing was about 2.5 times longer than it was on the less polar mixed phase column packing. Differences in the polarity and the peak elution patterns of these columns permitted the discrimination between authentic HCB and an unknown peak, which nearly coeluted with HCB on the mixed phase column.

Table III presents data from the analysis of feces samples from controls and rats treated with 20 mg lindane/kg/day for

Table I. Recovery of HCB and Lindane from Fecal Samples

Feces (mg)	Added Pesticide (ng)		Recovered Pesticide (ng)		Corrected % Recovery*	
	HCB	γ-HCH	HCB	γ-HCH	HCB	γ-HCH
391	59.3	101.7	50.5	89.6	85.2	85.9
371	59.3	101.7	50.4	91.0	85.0	87.4
602	59.3	101.7	48.8	91.0	82.4	87.4
494	59.3	101.7	52.0	93.7	87.6	90.0
495	59.3	101.7	46.8	88.6	78.9	84.9
480	59.3	101.7	54.6	89.8	92.1	86.2
430	0.0	0.0	—	2.2	—	—
					Mean ± SE 85.8 ± 1.8	87.0 ± 0.7

*The % recovery was corrected for the trace quantity of lindane found in the unspiked control feces.

six days. A peak in the vicinity of HCB was detected on the mixed phase column but not on the 5% EGS column. Although the analytical processor identified this peak as HCB, the retention times of the peak and standard HCB were not identical (4.18 vs. 4.39 min.). When calculated as HCB, there was no signficant difference between control and treated rats in the fecal content of this unknown. It thus appeared unlikely that the unidentified peak was derived from lindane. Comparative chromatography indicated that retention time of the unknown peak was different from that of several early eluting lindane metabolites (Table IV). The minimum detectable level for HCB in feces was determined to be 0.003 ppm on the 1.5% OV-17 + 1.95% QF-1 column packing and 0.008 ppm on the 5% EGS.

Representative chromatograms of the fecal extract from a treated rat are presented in Figure 1 before and after spiking with HCB. On the mixed phase column after addition of HCB, there was a slight alteration in the retention time of the peak which nearly coeluted with HCB, but no peak separation occurred. On the EGS column after the sample was spiked, a new peak appeared with a retention time identical to that of the HCB standard.

No HCB was detected in adipose tissue extracts on either column (Figure 2). After spiking the extracts with 590 pg of HCB, a peak appeared at the identical retention time of the HCB standard. The minimum detectable level for HCB in adipose tissue was determined to be 0.012 ppm on the mixed phase QF-1 column packing and 0.026 ppm on the 5% EGS.

The lindane content of the adipose tissue is presented in Table V. While the controls had trace quantities of lindane in their fat, rats treated with 20 mg lindane/kg/day for six days stored an average 129 ppm lindane in their adipose tissue.

Table II. Retention Times* of HCB and γ-HCH on Two Packed Columns

Pesticide	5% EGS	1.5% OV-17 + 1.95% QF-1
HCB	3.14 (0.181)	4.39 (0.651)
γ-HCH	17.31 (1.00)	6.74 (1.00)

*Absolute retention times in min are presented. Numbers in parentheses represent the retention time relative to lindane.

Discussion

Results from this study indicated that no significant biotransformation of lindane to hexachlorobenzene occurred in young adult female Fischer 344 rats. Gas-liquid chromatographic analysis of fat (Figure 2, Table V) and feces (Figure 1, Table III) extracts, from controls and rats treated with 20 mg lindane/kg/day for six days, indicated that, if present, the HCB content was less than the minimum detectable level (MDL) on two different column packings. For fat and feces the MDLs were 0.012 and 0.008 ppm, respectively. While no HCB was detected in adipose tissue on either column, the analytical processor indicated that a peak, which chromatographed at 4.18 min on the mixed phase column, represented a trace quantity of HCB in the feces extracts. Other considerations indicated that this identification was probably in error. Though the retention time of this peak did not exactly match that of the standard hexachlorobenzene (4.39 min), it was identified as HCB because it fell within the ±5% range set for the retention time window on the analytical processor. On the other hand, the analytical processor did not detect any HCB when the fecal extracts were analyzed on the 5% EGS column, indicating that the unidentified peak was not HCB. Moreover, even when the unknown was calculated as HCB (Table III), the trace quantity excreted in the feces from the treated rats was not signficantly different from that present in feces from control animals, making it unlikely that the unknown was derived from lindane. Comparison of the retention times of some early eluting lindane metabolites with that of the unknown (Table IV) failed to identify it.

Results from this study do not agree with those from the work of Gopalaswamy and Aiyar (1). They detected HCB in the feces of rats treated with lindane. However, details regarding the collection, storage, and extraction of the feces samples were not presented. Since it has been reported that microorganisms are capable of metabolizing lindane to HCB (19), the possible microbial transformation of lindane to HCB in fecal matter should not be ignored. Conceivably, fecal samples from lindane-treated rats, which were neither refrigerated nor extracted soon after collection, could contain significant quantities of HCB that would not be derived from the metabolism of lindane by the

Table III. HCB and γ-HCH Content* of Feces After Six Days of Treatment with 20 mg Lindane/kg

			Analysis on OV-17 + QF-1***		Analysis on EGS†	
Rat No.	Treatment**		HCB	γ-HCH	HCB	γ-HCH
25-C	Control		0.004	0.011	–	–
27-C	Control		0.006	0.010	–	–
36-C	Control		0.010	0.011	–	–
		Mean ± SE	0.007 ± 0.002	0.011 ± 0.0003		
1-L	Lindane		0.036	0.201	–	0.209
10-L	Lindane		0.004	1.35	–	1.27
11-L	Lindane		0.015	1.19	–	1.23
14-L	Lindane		0.010	1.41	–	1.46
15-L	Lindane		0.026	0.627	–	0.582
23-L	Lindane		0.004	2.10	–	2.21
		Mean ± SE	0.016 ± 0.005	1.15 ± 0.27	–	1.16 ± 0.29

*Values are ppm in feces.
**Treatment described in Experimental section.
***The lowest detection level for HCB on this column was determined to be 0.003 ppm.
†The lowest detection level for HCB on this column was determined to be 0.008 ppm. Only feces extracts from the treated rats were analyzed on this column.

rat. Furthermore, Gopalaswamy and Aiyar did not include any data on the extracts from control feces which would have reduced the chance that the peak identified as HCB was an artifact. These workers also reported the *in vitro* metabolism of γ-HCH to HCB in liver 9000-g supernatant following incubation with lindane. Extracts from the liver preparations were analyzed on a column packing almost identical to the one employed in this study, on which an interfering, unidentified peak eluted at a retention time close to that of HCB. Finally, since female Fischer 344 rats were used in this study while male

Table IV. Comparative Retention Times of Select Chemicals*

Chemical Identification**	Retention Times (Min)	Relative Retention Times
2,3,4,6-Tetrachloro-2-cyclohexen-1-ol	2.50	0.37
γ-1,2,3,4,5,6-Hexachlorocyclohex-1-ene	2.99	0.44
2,4,5,6-Tetrachloro-2-cyclohexen-1-ol	3.14	0.47
Unknown Peak	4.18	0.62
2,3,4,5,6-Pentachloro-2-cyclohexen-1-ol	4.34	0.64
Hexachlorobenzene (HCB)	4.39	0.65
α-Hexachlorocyclohexane	5.02	0.74
γ-Hexachlorocyclohexane (Lindane)	6.74	1.00

*Retention times were determined on a column packed with 1.5% OV-17 + 1.95% QF-1 on 80/100 mesh Gas Chrom Q.
**All chemicals other than hexachlorobenzene, γ-hexachlorocyclohexane (lindane) and α-hexachlorocyclohexane are known lindane metabolites.

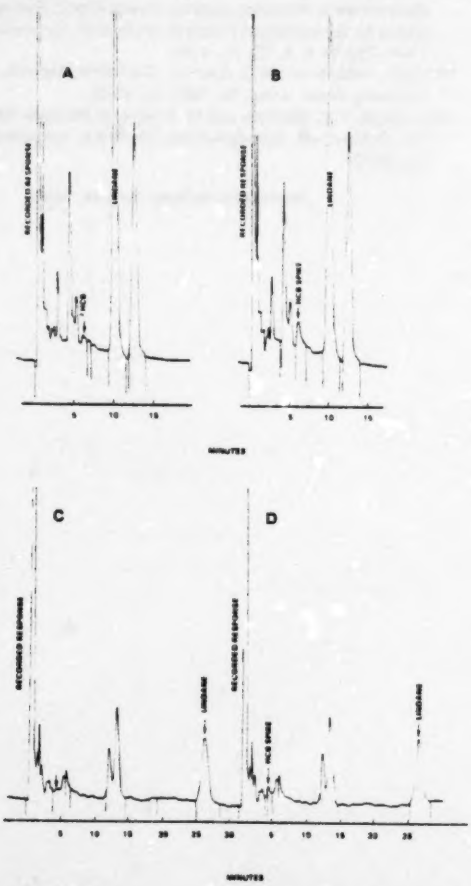

Figure 1. GLC chromatograms of a representative fecal extract on 1.5% OV-17+1.95% QF-1 before (A) and after (B) addition of 590 pg HCB and on 5% EGS before (C) and after (D) addition of 590 pg HCB.

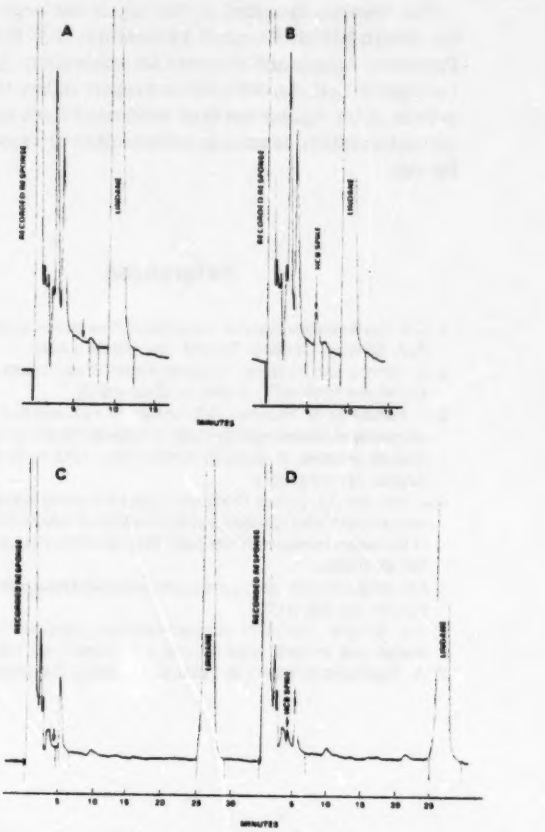

Figure 2. GLC chromatograms of a representative adipose tissue extract on 1.5% OV-17+1.95% QF-1 before (A) and after (B) addition of 590 pg HCB and on 5% EGS before (C) and after (D) addition of 590 pg HCB.

Table V. Lindane in the Adipose Tissue of Rats Treated with 20 mg γ-HCH/kg/day for Six Days

Rat Identification	Treatment	Lindane Content (ppm in Lipid)
25-C	Control	0.04
27-C	Control	0.04
36-C	Control	0.05
	Mean ± SE	0.04 ± 0.003
1-L	Lindane	136.55
10-L	Lindane	155.76
11-L	Lindane	118.03
14-L	Lindane	135.74
15-L	Lindane	112.07
23-L	Lindane	118.92
	Mean ± SE	129 ± 6

*This analysis was conducted on the column packed with 1.5% OV-17 + 1.95% QF-1 on 80/100 mesh Gas Chrom Q.

Wistar rats were used by Gopalaswamy and Aiyar, both strain and sex differences could have contributed to the differences observed between these studies.

Disclaimer

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